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Ciliary Neurotrophic Factor Protects Mice Against Streptozotocin-induced Type 1 Diabetes through SOCS3

THE ROLE OF STAT1/STAT3 RATIO IN β-CELL DEATH[*S]

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Background: CNTF promotes islet survival, possibly protecting mice against type 1 diabetes.
Results: CNTF inhibits STZ- and IL1β-induced apoptosis of islets and increases SOCS3 expression.
Conclusion: CNTF protects against STZ-induced diabetes, which depends on increased SOCS3 expression and reduced STAT1/STAT3 ratio.
Significance: Understanding the mechanisms that determine pancreatic islet fate is crucial for the prevention and treatment of diabetes.

Type 1 diabetes is characterized by a loss of islet β-cells. Ciliary neurotrophic factor (CNTF) protects pancreatic islets against cytokine-induced apoptosis. For this reason, we assessed whether CNTF protects mice against streptozotocin-induced diabetes (a model of type 1 diabetes) and the mechanism for this protection. WT and SOCS3 knockdown C57BL6 mice were treated for 5 days with citrate buffer or 0.1 mg/kg CNTF before receiving 80 mg/kg streptozotocin. Glycemia in non-fasted mice was measured weekly from days 0–28 after streptozotocin administration. Diabetes was defined as a blood glucose > 11.2 mmol/liter. Wild-type (WT) and SOCS3 knockdown MIN6 cells were cultured with CNTF, IL1β, or both. CNTF reduced diabetes incidence and islet apoptosis in WT but not in SOCS3kd mice. Likewise, CNTF inhibited apoptosis in WT but not in SOCS3kd MIN6 cells. CNTF increased STAT3 phosphorylation in WT and SOCS3kd mice and MIN6 cells but reduced STAT1 phosphorylation only in WT mice, in contrast to streptozotocin and IL1β. Moreover, CNTF reduced NFκB activation and required down-regulation of inducible NO synthase expression to exert its protective effects. In conclusion, CNTF protects mice against streptozotocin-induced diabetes by increasing pancreatic islet survival, and this protection depends on SOCS3. In addition, SOCS3 expression and β-cell fate are dependent on STAT1/STAT3 ratio.

Type 1 diabetes is a complex illness that ultimately abrogates the capacity of the organism to produce and secrete insulin. This disorder is characterized by severe hyperglycemia as a consequence of the selective loss of pancreatic β-cells, mainly induced by inflammatory cytokines, especially IL1β. The mechanisms underlying IL1β-induced β-cell death are not fully understood but appear to involve the activation of the STAT1 (1) and NFκB (2) pathways, which in turn control iNOS[2] expression (2). These events then lead to apoptosis, which plays a major role in β-cell death and, therefore, type 1 diabetes onset and development (3, 4).

CNTF is a member of the IL6 family of cytokines, which includes IL11, leukemia inhibitory factor, cardiotrophin-1, oncostatin-M, CNTF, and IL6 itself, all using gp130 as a signal-transducing element in the functional receptor complexes and a specific receptor for each of them (5). CNTF is distributed throughout the rat central and peripheral nervous system, in neurons, glial, and Schwann cells (6, 7), and acts as a survival factor for neurons (8) and pancreatic islets (9). Although CNTF impairs glucose-stimulated insulin secretion (9, 10), it is anti-diabetogenic and exhibits many *in vivo* systemic effects, such as a reduction in adiposity, body weight, hyperinsulinemia, and hyperglycemia in rats (11–18).

In pancreatic islets, CNTF signals through the JAK2/STAT3 (19). Binding of CNTF to CNTF Receptor on the gp130 complex activates the receptor-associated kinase JAK2 (20) and phosphorylates tyrosine residues on CNTF Receptor, recruiting and phosphorylating STAT3, which dimerizes and translocates to the nucleus to regulate gene transcription (5, 21). STAT3 activation leads to cell differentiation, migration, and inhibition of apoptosis and is therefore described as an anti-inflammatory, anti-apoptotic, and prosurvival pathway, in opposition to the inflammatory, apoptotic and death-inducer role of the STAT1 pathway (22, 23).

Regulation of the STAT pathway involves multiple mechanisms, particularly increased expression suppression of cytokine signaling 3 (SOCS3) (24). SOCS3 protects pancreatic islets from IL1β-induced toxicity (25, 26), inhibits streptozotocin-induced type 1 diabetes (27), and regulates β-cell mass and proliferation (28), differential gene expression (29), and insulin

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[2] The abbreviations used are: iNOS, inducible NO synthase; CNTF, ciliary neurotrophic factor; SOCS3, suppressor of cytokine signaling 3; STZ, streptozotocin; kd, knockdown.

secretion (30). Despite these promising effects, SOCS3 is invariably expressed as a negative feedback signal after the exposure of cells to inflammatory cytokines, a fact that limits the potential of SOCS3 as a pharmacological target.

We have shown that CNTF not only promotes rat pancreatic islet survival (9) but also protects rat pancreatic islets and MIN6 cells against IL1β-induced apoptosis. Furthermore, CNTF-induced β-cell protection depends on JAK2/STAT3 pathway activation and increased SOCS3 expression (19). Because cytokine-induced β-cell apoptosis is an important event in the pathogenesis of type 1 diabetes and CNTF protects β-cells against IL1β-induced apoptosis, our primary goals in the present study was 1) to verify whether CNTF could protect mice against type 1 diabetes in a model that is heavily dependent upon inflammatory cytokine damage (streptozotocin-induced) and 2) to determine whether this protection depends upon increased SOCS3 expression in mice pancreatic islets.

EXPERIMENTAL PROCEDURES

Reagents—Streptozotocin was acquired from Sigma Aldrich. Recombinant rat interleukin-1β was from Invitrogen™. Western blot detection of specific proteins used the following primary antibodies: SOCS3, total STAT3, phosphorylated STAT3, total STAT1, phosphorylated STAT1, IκB-α, phosphorylated p65, iNOS, and GAPDH from Santa Cruz Biotechnology, and cleaved and intact caspase-3 from Cell Signaling Technology (Boston, MA). The secondary antibodies used were anti-rabbit IgG and anti-mouse IgG (Cell Signaling Technology). The urea anti-protease/anti-phosphatase buffer was composed of 7 M urea, 2 M thiourea, 5 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 2 mM PMSF, 1% Triton X-100, and 1 μg/ml aprotinin (Bayer Health Care Pharmaceuticals, Berkeley, CA).

Animals—The mice were obtained from the Central Animal Handling Facility at the State University of Campinas. Both wild-type and iNOS-knock-out mice were from a C57BL/6 background. Throughout the text, wild-type mice were designated as WT, and iNOS knock-out mice were designated as iNOS$^{-/-}$. SOCS3 knockdown mice (designated as SOCS3kd mice) received a daily intraperitoneal injection of 1 nmol of SOCS3-antisense oligonucleotide dissolved in tris-EDTA buffer plus JetPei-In (according to the manufacturer's instructions) for 2 days before and for 3 days after the start of CNTF treatment, totaling 5 consecutive days. The effectiveness of SOCS3 antisense compared with the SOCS3 mismatch oligonucleotide after 48 h and before CNTF treatment was evaluated by RT-PCR and Western blots (supplemental Fig. 1). All animals were male and six- to 8-weeks-old at the start of experiment. Throughout the duration of the experiment, animals were kept in individual cages with *ad libitum* access to food (standard chow diet) and water, in a 12/12-h light/dark cycle. Primers used were as follows: SOCS3 (antisense), mC*mC*mU*mC*mA*T*C*T*G*T*C*T*C*mC*mC*mU*mU*mC; SOCS3 (mismatch), mC*mC*mU*mC*mT*T*G*T*G*A*G*T*C*mC*mC*mU*mU*mG.

In Vivo Experimental Design and Pancreatic Islet Isolation—Initially, a group of wild-type C57BL/6 mice received daily intraperitoneal injections of CNTF (0.1 mg/kg dissolved in citrate buffer, pH 4.5) or vehicle (citrate buffer, pH 4.5). Six hours after the last dose of CNTF or vehicle, the mice received an intraperitoneal injection of streptozotocin (STZ) (80 mg/kg dissolved in citrate buffer, pH 4.5) or vehicle (citrate buffer, pH 4.5); these groups corresponded to the groups control (vehicle before vehicle), CNTF (CNTF before vehicle), STZ (vehicle before STZ), and CNTF+STZ (CNTF before STZ). Secondly, a group of SOCS3kd mice went through the same experimental procedures and were separated into groups S3 (SOCS3kd, vehicle before vehicle), S3.CNTF (SOCS3kd, CNTF before vehicle), S3.STZ (SOCS3kd, vehicle before STZ) and S3.CNTF+STZ (SOCS3kd, CNTF before STZ). Finally, the same treatments were applied to the iNOS$^{-/-}$ mice: iNOS, iNOS.CNTF, iNOS.STZ and iNOS.CNTF.STZ. Day 1 was considered as the day of the first CNTF injection, and mouse blood glucose was determined with a blood glucose meter (Accucheck Performa II) from a drop-sized sample from the caudal artery. Observations were performed on days 1 (before CNTF injection), 2, 3, 4, 5, 7, 14, 21, and 28 for non-fasted mice. Mice were considered to have diabetes when the non-fasted blood glucose was higher than 11.2 mmol/l for 2 consecutive days (supplemental Fig. 2).

Some mice were euthanized at 24 h after STZ administration in a CO_2-saturated atmosphere, immediately followed by decapitation, for organ harvesting and pancreatic islet isolation and collection using the collagenase method. Islet protein levels were measured by Western blot and mRNA levels using real-time RT-PCR.

In Vitro/MIN6 Cells, siRNA Transfection, and Experimental Design—MIN6 cells were transfected with siRNA directed against SOCS3 or scramble siRNA (Santa Cruz Biotechnology). Briefly, the cells were transfected with 200 nM of total siRNA using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. MIN6 insulin-producing cells (passages 30–45) were cultured in RPMI 1640, supplemented with 2% (v/v) of fetal calf serum-free, penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. Cells were plated at a density of 2×10^5 per 50-mm plastic dish. Subsequently, cells that were or were not transfected with siSOCS3 were exposed to 10 ng/ml of IL1β following (or not) pretreatment with 1 nM CNTF. The efficiency of siSOCS3 was evaluated by RT-PCR and Western blot demonstrating SOCS3 expression (supplemental Fig. 1).

Western Blot—The protein concentration was determined by the Bradford method using bovine serum albumin as the standard. Seventy μg of the lysate was boiled in SDS loading buffer and applied to 10% or 12% SDS-PAGE, transferred to nitrocellulose membranes, and stained with Ponceau. Membranes were blocked in 10 mmol/liter Tris base, 150 mmol/liter NaCl, and 0.25% (v/v) of Tween 20 (TBS buffer) containing 5% (w/v) low-fat milk powder for 1 h at room temperature. The membranes were then incubated with primary antibodies overnight at 4 °C. Detection was performed using enhanced chemiluminescence (SuperSignal West Pico, Pierce) after incubation with a horseradish peroxidase-conjugated secondary antibody. The band intensities were quantified by optical densitometry (Scion Image) of the developed autoradiogram.

Quantitative Real-time PCR—Groups of MIN6 cells were homogenized in Trizol® following phenol-chloroform RNA

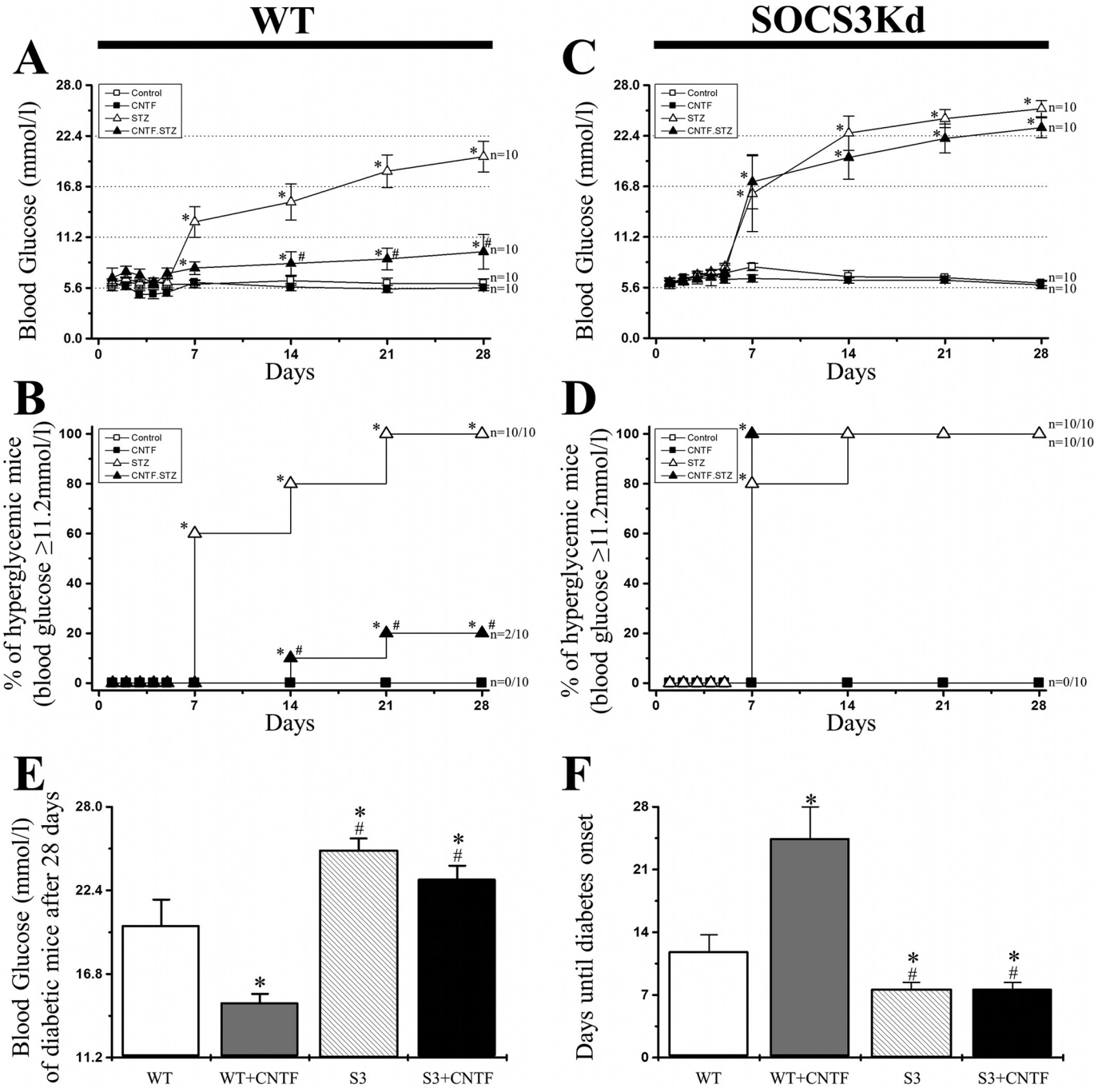


FIGURE 1. **CNTF protects wild-type but not SOCS3kd mice against STZ-induced diabetes.** Wild-type and SOCS3kd C57BL6 mice were treated with citrate buffer (*white squares*), 0.1 mg/kg CNTF (*black squares*), 80 mg/kg streptozotocin (*white triangles*), or both (*black triangles*). Diabetes was considered when blood glucose of non-fasted mice ≥ 11.2mmol/liter for two consecutive days. Blood glucose (mmol/liter; *A*) and diabetes incidence (%; *B*) of wild-type mice are shown. Blood glucose (mmol/liter; *C*) and diabetes incidence (%; *D*) of SOCS3kd mice are shown. *E*, blood glucose (mmol/liter) of wild-type and SOCS3kd mice with diagnosed type 1 diabetes 28 days after streptozotocin administration. *F*, days after streptozotocin administration until type 1 diabetes onset in wild-type and SOCS3kd (*S3*) mice. *White bars*, control; *gray bars*, CNTF; *hatched bars*, streptozotocin; and *black bars*, CNTF + streptozotocin ($n = 10$). Data are means ± S.E. *, significantly different from control. #, significantly different from CNTF. †, significantly different from streptozotocin.

extraction, according to the manufacturer's instructions. The RNA integrity was examined by agarose gel, and its concentration was measured by GeneQuant (PharmaciaBiotec). The following reverse transcriptase PCR for cDNA synthesis was made using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Relative quantification was performed using the Step one real-time PCR systems (Applied Biosystems). The primers were designed using Primer Express software (Applied Biosystems). The primers were designed and tested against the *Mus musculus* genome (Gene Bank) to ensure that no amplification of other cDNAs had occurred. Relative quantities of target transcripts were calculated from duplicate samples after the data were normalized against the endogenous control β-actin. The primers used were as follows: SOCS3-F, 5′-GGAGGGTTCTGCTTTGTC-3′; SOCS3-R, 5′-GTGTTTGGCTCCTTGTGTGC-3′; iNOS-F,

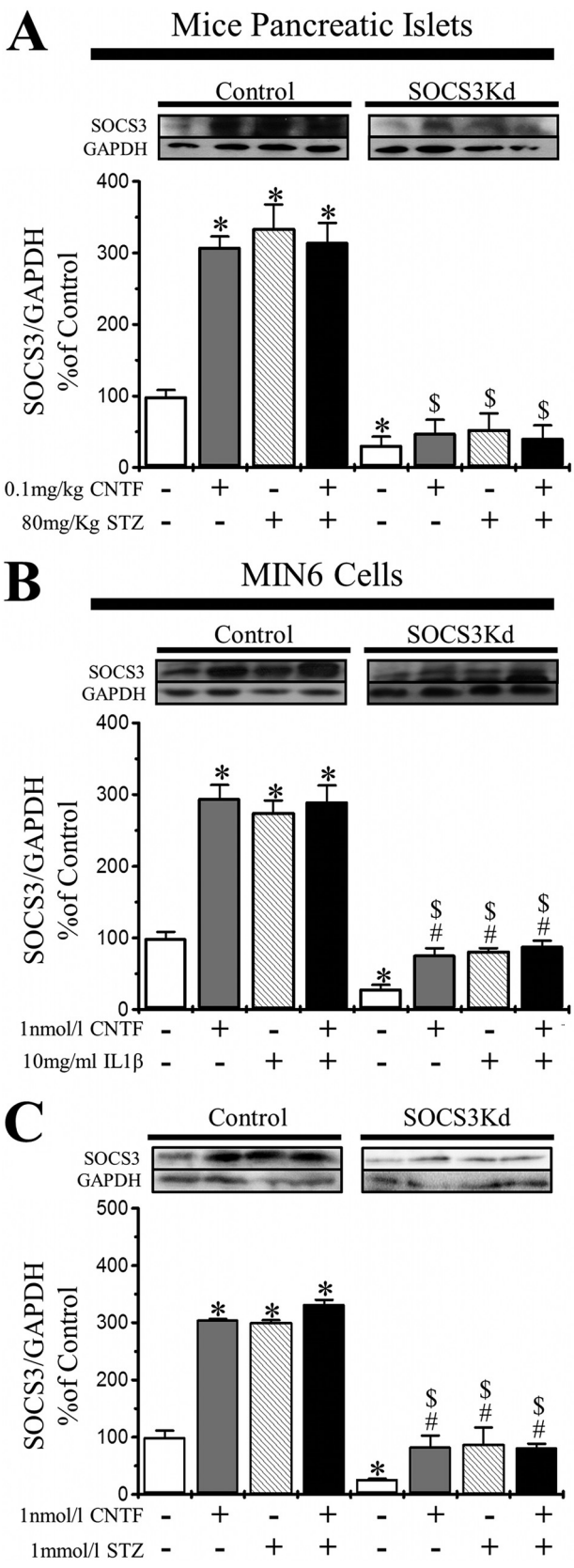


FIGURE 2. **CNTF, STZ, and IL1β increased SOCS3 expression on islets and MIN6 cells.** Shown is the SOCS3 expression of pancreatic islets from wild-type and SOCS3kd C57BL6 mice treated with citrate buffer (*white bars*), 0.1 mg/kg of CNTF (*gray bars*), 80 mg/kg of streptozotocin (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from streptozotocin; $, significantly different from respective wild-type group. Shown is the SOCS3 expression of wild-type and SOCS3kd MIN6 cells treated with citrate buffer (*white bars*), 1 nmol/liter CNTF (*gray bars*), 10 ng/ml IL1β (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from IL1β; $, significantly different from respective wild-type group. Shown is the SOCS3 expression of wild-type and SOCS3kd MIN6 cells treated with citrate buffer (*white bars*), 1 nmol/liter of CNTF (*gray bars*), 1mmol/liter STZ (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from STZ; $, significantly different from respective wild-type group.

5′-CCACGGACGAGACGGATAGGC-3′; iNOS-R, 5′-GCAGGAAGGCAGCGGGCACA-3′; β-actin-F, 5′-AGAGGGAAATCGTGCGTGACA-3′; and β-actin-R, 5′-CGATAGTGATGACCTGACCGTCA-3′.

DNA Fragmentation Assay—DNA was isolated from MIN6 cells after treatment and separated into fragmented and integral subunits by the TRIzol/Triton method. Both were quantified by the SYBR-green method as ng/ml of DNA. The data are expressed as fragmented/total DNA percentage.

Statistics—All data are expressed as the means ± S.E. Statistical analyses were performed using Student's *t* test or one-way ANOVA followed by Bonferroni's or Dunnett's test. A value of $p < 0.01$ was considered statistically significant for PCR experiments and $p < 0.05$ for other experiments.

RESULTS

Although all of the STZ-treated WT C57BL/6 control mice developed hyperglycemia (Fig. 1*A*) and overt diabetes (Fig. 1*B*), only 20% of the STZ-treated WT C57BL/6 CNTF mice developed hyperglycemia (Fig. 1*A*) and diabetes (Fig. 1*B*) after 28 days. Furthermore, the mice treated with CNTF that became diabetic presented less severe hyperglycemia (Fig. 1*E*) and a delayed onset of the disease (Fig. 1*F*). As such, CNTF delays, ameliorates, and protects mice against STZ-induced diabetes. C57BL/6 STZ-treated mice that were knocked down for SOCS3 (SOCS3kd) developed more severe hyperglycemia (Fig. 1*E*), which was established earlier (Fig. 1*F*) than in the STZ-treated control mice. Pretreatment of SOCS3kd mice with CNTF failed to prevent (Fig. 1, *C* and *D*), ameliorate (Fig. 1*E*), or delay (Fig. 1*F*) STZ-induced diabetes, showing that the protective effects of CNTF depend on SOCS3 expression.

Because CNTF protects mice against STZ-induced type 1 diabetes and because this effect depends upon SOCS3, we evaluated how CNTF, STZ, and CNTF+STZ regulate SOCS3 expression in pancreatic islets from WT C57BL/6 and SOCS3kd mice. In addition, we investigated how CNTF, IL1β, STZ, CNTF+IL1β, and CNTF+STZ regulate SOCS3 expression in WT and SOCS3kd MIN6 cells. We found that CNTF, STZ, and CNTF+STZ promote a 3- to 4-fold increase in SOCS3 expression in pancreatic islets from WT mice but not from SOCS3kd mice (Fig. 2*A*). CNTF and IL1β, alone or in combination, induced a 2- to 3-fold increase in SOCS3 expression in WT MIN6 cells but not in SOCS3kd MIN6 cells (Fig. 2*B*). Similarly, CNTF and STZ, alone or in combination, increased SOCS3 expression in WT MIN6 cells but not in SOCS3kd MIN6 cells (Fig. 2*C*) These results provided a link between the effects of CNTF on pancreatic islets from STZ-

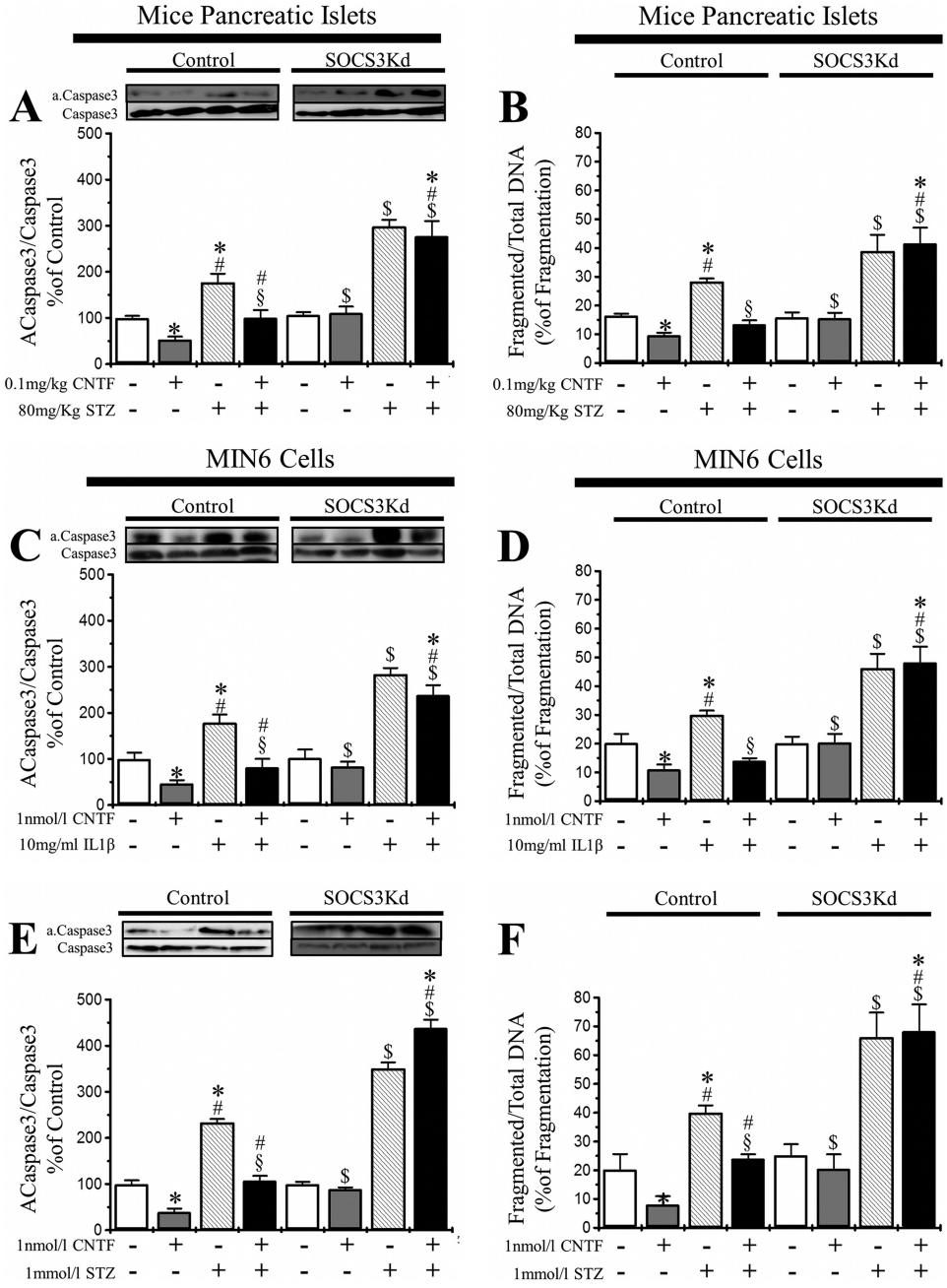


FIGURE 3. **CNTF prevents apoptosis from WT but not from SOCS3kd islets and MIN6 cells.** Caspase-3 cleavage (*A*) and DNA fragmentation (*B*) of pancreatic islets from wild-type and SOCS3kd C57BL6 mice treated with citrate buffer (*white bars*), 0.1 mg/kg of CNTF (*gray bars*), 80 mg/kg of streptozotocin (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from streptozotocin; $, significantly different from respective wild-type group. Shown are caspase-3 cleavage, meant as Activate Caspase 3 (ACaspase3)/Caspase3 ratio (*C*) and DNA fragmentation (*D*) of wild-type and SOCS3kd MIN6 cells treated with citrate buffer (*white bars*), 1 nmol/liter of CNTF (*gray bars*), 10 ng/ml of IL1β (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from IL1β; $, significantly different from respective wild-type group. Shown are caspase-3 cleavage, meant as Activate Caspase 3 (ACaspase3)/Caspase3 ratio (*E*) and DNA fragmentation (*F*) of wild-type and SOCS3kd MIN6 cells treated with citrate buffer (*white bars*), 1 nmol/liter CNTF (*gray bars*), 1 mmol/liter STZ (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from STZ; $, significantly different from respective wild-type group.

induced type 1 diabetic mice and the effects of CNTF on mouse β-cells treated with IL1β.

As mentioned previously, type 1 diabetes resulted, at least in part, from cytokine-induced β-cell apoptosis. Therefore, we evaluated the effects of CNTF on the apoptosis of pancreatic islets from WT and SOCS3kd mice, as well as its effects on WT and SOCS3kd MIN6 cells. CNTF reduced apoptosis in pancreatic islets from WT mice and WT MIN6 cells, but not in pancreatic islets from SOCS3kd mice and SOCS3kd MIN6 cells. Moreover, CNTF prevented STZ-induced apoptosis in pancreatic islets from WT mice but not in pancreatic islets from SOCS3kd mice (Fig. 3, *A* and *B*). CNTF prevented IL1β-induced apoptosis in WT MIN6 cells but not in SOCS3kd MIN6 cells (Fig. 3, *C* and *D*). Likewise, CNTF prevented STZ-induced

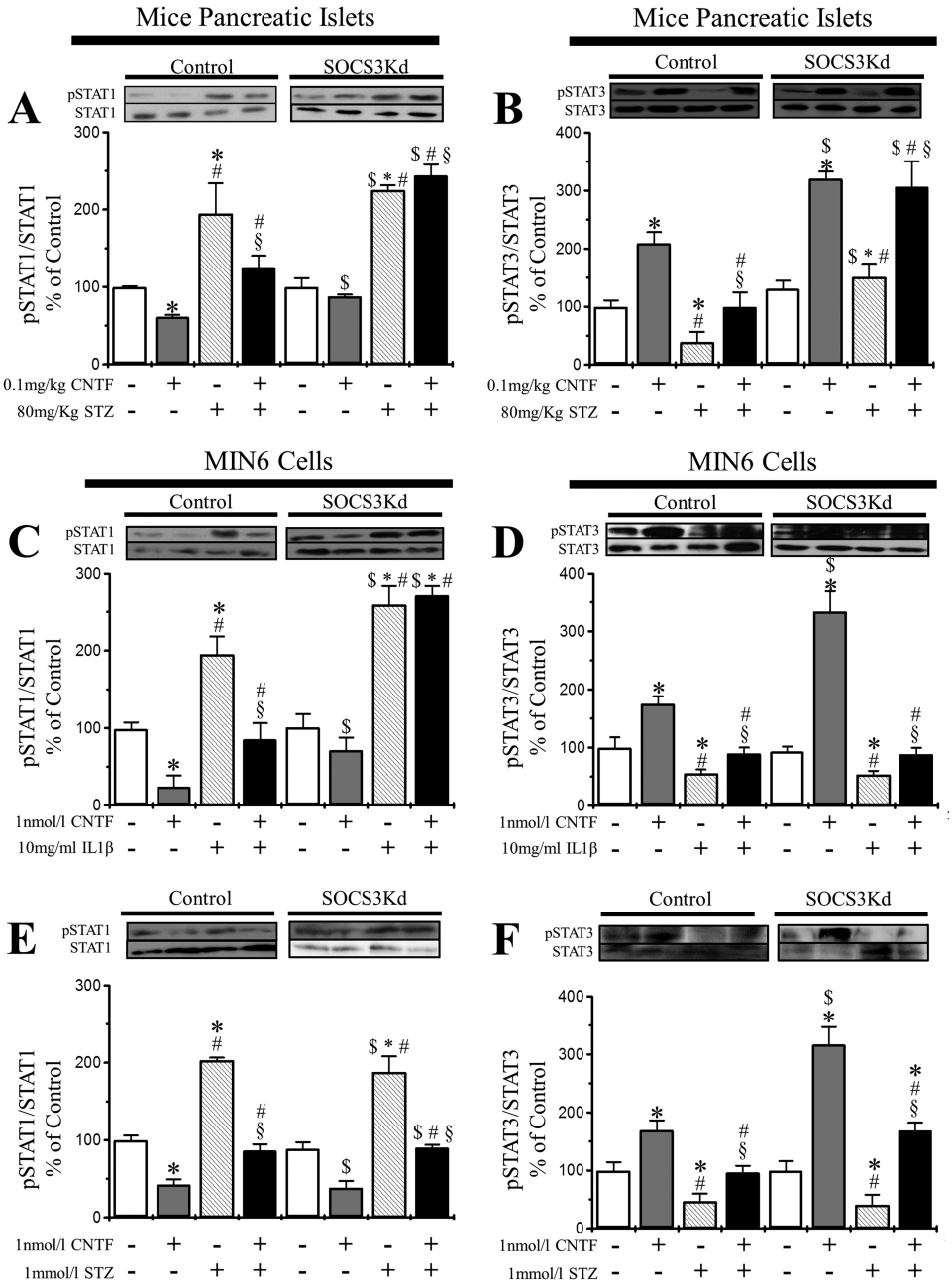


FIGURE 4. **CNTF inhibits STAT1 and promotes STAT3 phosphorylation in WT islets and cells but was unable inhibit STAT1 in SOCS3kd islets and cells.** STAT1 (*A*) and STAT3 (*B*) phosphorylation of pancreatic islets from wild-type and SOCS3kd C57BL6 mice treated with citrate buffer (*white bars*), 0.1 mg/kg of CNTF (*gray bars*), 80 mg/kg of streptozotocin (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from streptozotocin; $, significantly different from respective wild-type group. Shown are STAT1 (*C*) and STAT3 (*D*) phosphorylation of wild-type and SOCS3kd MIN6 cells treated with citrate buffer (*white bars*), 1 nmol/liter of CNTF (*gray bars*), 10 ng/ml of IL1β (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from IL1β; $, significantly different from respective wild-type group. Shown are STAT1 (*E*) and STAT3 (*F*) phosphorylation of wild-type and SOCS3kd MIN6 cells treated with citrate buffer (*white bars*), 1 nmol/liter CNTF (*gray bars*), 1mmol/liter STZ (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from STZ; $, significantly different from respective wild-type group.

apoptosis in WT MIN6 cells, but not in SOCS3kd MIN6 cells (Fig. 3, *E* and *F*). These results provide further evidence that CNTF protects against STZ-induced type 1 diabetes, at least in part, by preventing apoptosis and promoting the survival of pancreatic islet β-cells. Furthermore, this protective effect requires increased SOCS3 expression.

Given that CNTF inhibited the apoptotic effects of STZ and IL1β, but that all of these agents increased SOCS3 expression, we assessed STAT1 and STAT3 phosphorylation, the major upstream regulators of SOCS3 expression. CNTF increased STAT3 phosphorylation in pancreatic islets from both WT and SOCS3kd mice, as well as in WT and SOCS3kd MIN6 cells. In addition, the increase in STAT3 phosphorylation was higher in SOCS3kd than in WT islets and MIN6 cells. CNTF inhibited STAT1 phosphorylation in WT mice pancreatic islets and MIN6 cells but not in SOCS3kd pancreatic islets and MIN6 cells (Fig. 4).

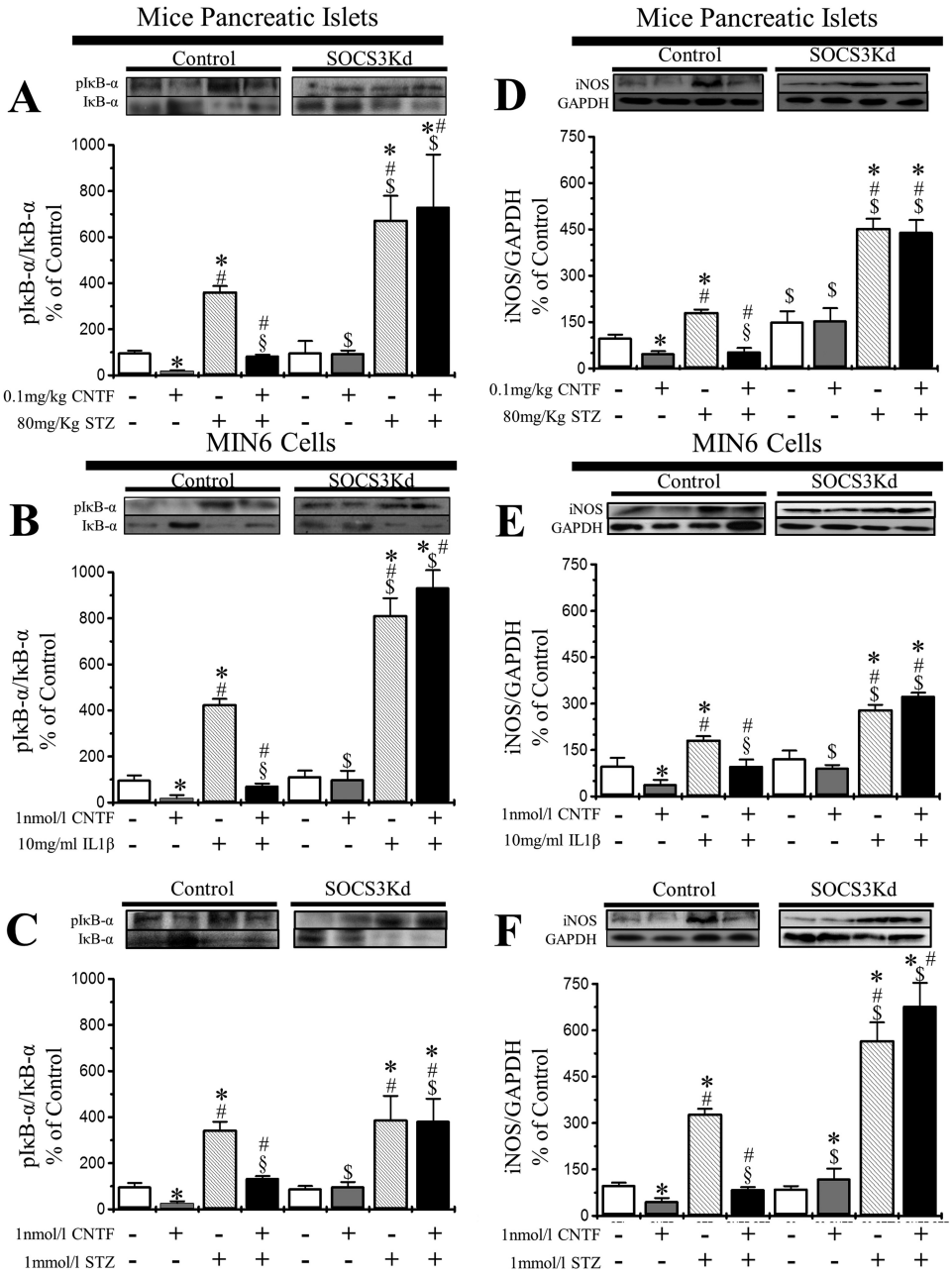


FIGURE 5. **CNTF inhibits IL1β- and STZ-induced NFκB pathway activation and iNOS expression in WT but not in SOCS3kd islets and MIN6 cells.** Shown are IκB-α phosphorylation (*A*) and iNOS expression (*B*) on pancreatic islets from wild-type and SOCS3kd C57BL6 mice treated with citrate buffer (*white bars*), 0.1 mg/kg CNTF (*gray bars*), 80 mg/kg streptozotocin (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from streptozotocin; $, significantly different from respective wild-type group. Shown are IκB-α phosphorylation (*C*) and iNOS expression (*D*) on wild-type and SOCS3kd MIN6 cells treated with citrate buffer (*white bars*), 1 nmol/liter CNTF (*gray bars*), 10 ng/ml IL1β (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from IL1β; $, significantly different from respective wild-type group. Shown are IκB-α phosphorylation (*E*) and iNOS expression (*F*) on wild-type and SOCS3kd MIN6 cells treated with citrate buffer (*white bars*), 1 nmol/liter CNTF (*gray bars*), 1 mmol/liter STZ (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from STZ; $, significantly different from respective wild-type group.

Because CNTF controls STAT1 and STAT3 activity in pancreatic islets and MIN6 cells and these proteins modulate the NFκB pathway, we evaluated the activation of this transcription factor. CNTF alone inhibited NFκB activation and prevented STZ-induced NFκB activation in pancreatic islets from WT mice but not SOCS3kd mice (Fig. 5*A*). CNTF alone inhibited NFκB activation and prevented both IL1β-induced (Fig. 5*B*) and STZ-induced (Fig. 5*C*) NFκB activation in WT MIN6 cells but not in SOCS3kd MIN6 cells (Fig. 5*B*).

Apoptosis of β-cells in type 1 diabetes involves increased expression of iNOS, which is controlled, at least in part, by NFκB. Thus, we evaluated the effects of CNTF on iNOS expression in WT and SOCS3kd pancreatic islets and MIN6 cells. CNTF prevented STZ-induced iNOS expression in pancreatic islets from WT mice but not in SOCS3kd mice (Fig. 5*D*). Likewise, CNTF prevented both IL1β-induced (Fig. 5*E*) and STZ-induced (Fig. 5*F*) iNOS expression in WT MIN6 cells but not in SOCS3kd MIN6 cells. The results presented here provide evi-

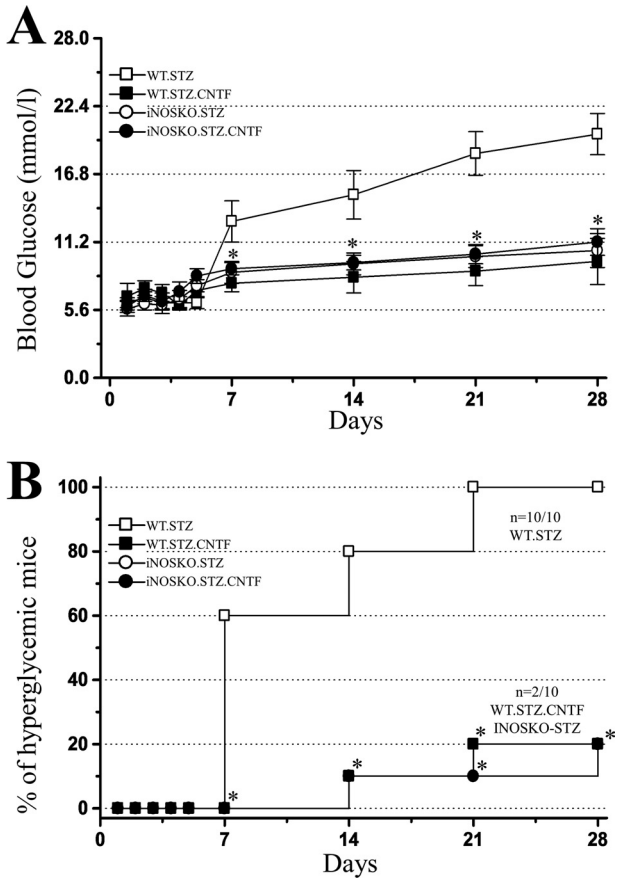


FIGURE 6. **CNTF was unable to further protect iNOSko mice against STZ-induced diabetes.** Wild-type, SOCS3kd, and iNOS$^{-/-}$ C57BL6 mice treated with citrate buffer (*white squares*), 0.1 mg/kg CNTF (*black squares*), 80 mg/kg streptozotocin (*white triangle*), or both (*black triangle*). Diabetes was considered when blood glucose of non-fasted mice ≥ 11.2mmol/liter for two consecutive days. Blood glucose (mmol/liter; *A*) and diabetes incidence (% of hyperglycemic mice; *B*) of wild-type and iNOSko mice.

dence that the protection exerted by CNTF in pancreatic islets and β-cells requires not only STAT3 activation but also the inhibition of STAT1 through increased SOCS3 expression, as well as reduced iNOS expression via NFκB down-regulation.

Our next step was to evaluate the effects of CNTF on iNOSko mice and MIN6 cells treated with *N*-Nitro-L-Arginine Methyl Ester. iNOSko mice had lower glycemia and a reduced incidence of diabetes after STZ administration compared with the WT mice, and CNTF was unable to further protect the iNOSko mice against STZ-induced diabetes (Fig. 6).

Thus, we assessed these pathways in pancreatic islets from WT and iNOS$^{-/-}$ mice, as well as from MIN6 cells treated or not with STZ in the presence of *N*-Nitro-L-Arginine Methyl Ester. The effects of CNTF on pathways upstream of iNOS were similar in WT and iNOSko mice: increased SOCS3 expression (Fig. 7*A*), increased STAT3 phosphorylation (Fig. 7*B*), reduced STAT1 phosphorylation (Fig. 7*C*), and reduced IκB-α phosphorylation (Fig. 7*D*). However, no difference in apoptosis was observed between the pancreatic islets of WT and iNOSko mice (Fig. 7*E*), providing further evidence that iNOS is the effector required for anti-apoptotic effects of CNTF *in vivo*. The effects of CNTF on signaling pathways were the same in MIN6 cells that were treated or not with *N*-Nitro-L-Arginine Methyl Ester (Fig. 7, *F–I*); however, contrary to the *in vivo* results, STZ still induced apoptosis *in vitro* (Fig. 7*H*).

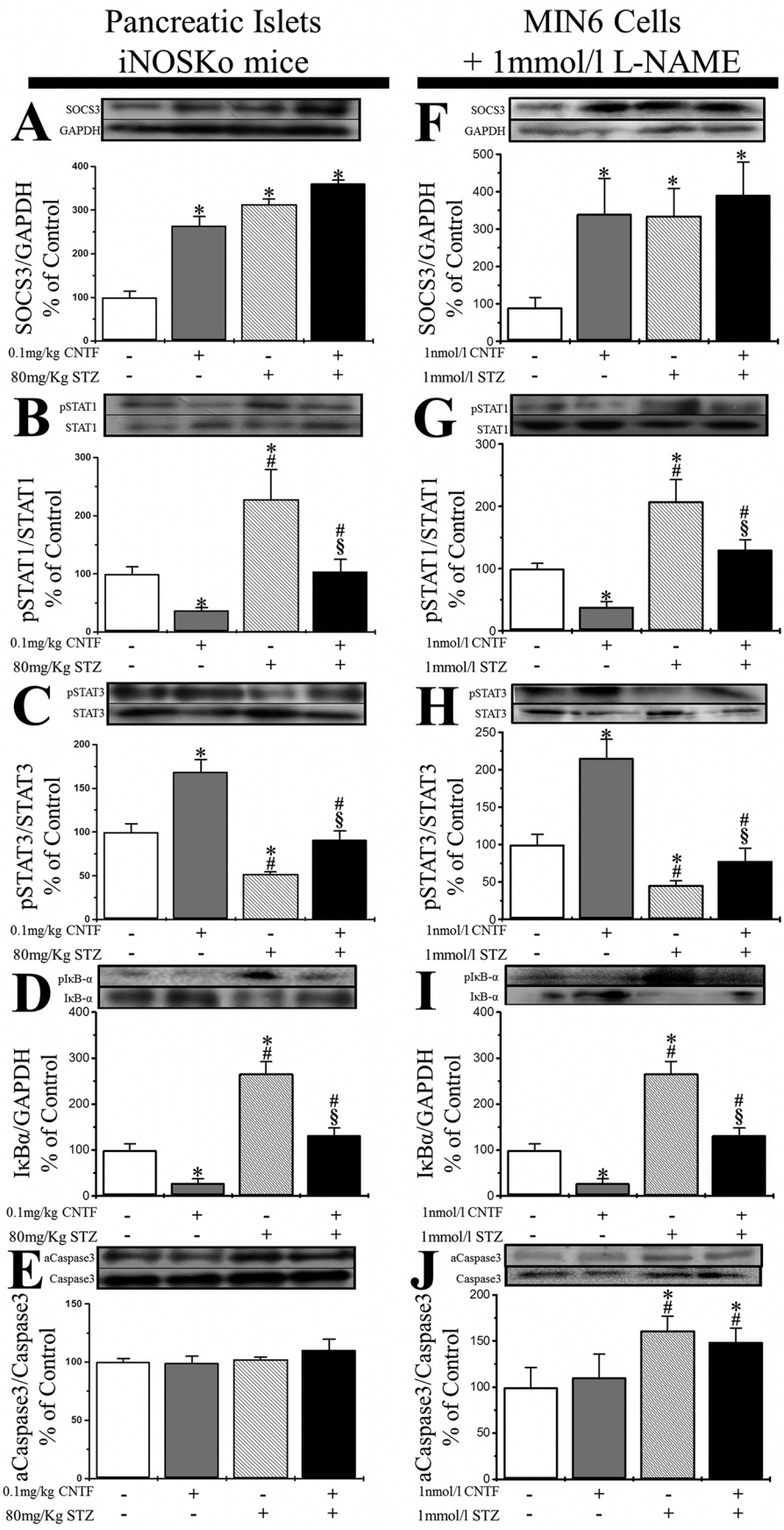
DISCUSSION

Type 1 diabetes onset and development are not yet completely understood, but it is well known that this disease depends on genetic and environmental factors, with an exogenous trigger being capable of setting off the initial failure in the immune system, which then incorrectly recognizes pancreatic β-cells as foreign cells. At this point, there was an increase in cytokine release and immune cells in the pancreatic islets, leading β-cells to undergo apoptosis, and finally to a complete loss of β-cell mass, insulin deficiency, and hyperglycemia (4, 31). Although this process is not completely understood, the mechanism of pancreatic β-cell death in type 1 diabetes apparently involves the action of three cytokines, IL1β, TNFα, and IFNγ, and even their relative contribution to β-cell apoptosis in type 1 diabetes or its intracellular mechanisms for this effect remain controversial.

For this reason, effective approaches to prevent or ameliorate the pathogenesis of type 1 diabetes are elusive (32). In this regard, our group recently demonstrated that CNTF, an anti-inflammatory cytokine belonging to the IL6 family, protects rat pancreatic islets (9, 19) and mice β-cells (33) against IL1β-induced apoptosis, making it a candidate for protection against STZ-induced type 1 diabetes. Here, we show that CNTF indeed prevented, delayed, and ameliorated STZ-induced type 1 diabetes in mice, an effect dependent upon increased SOCS3 expression in pancreatic islets.

The role of SOCS3 in β-cell death and the development of type 1 diabetes has been extensively studied in recent years, although with conflicting results. SOCS3 expression inhibits IL1-induced apoptosis in primary pancreatic islets from the mouse and rat (19, 25, 26, 28, 34), whereas SOCS3 knock-out mice have been found to be resistant to streptozotocin-induced type 1 diabetes (27). Our results provide evidence that, although seemingly contradictory, the previous findings regarding the role of SOCS3 in pancreatic islets represented the same phenomenon only in a complementary manner. STAT1 activation by apoptotic cytokines (IL1β) as well as STAT3 activation by an anti-apoptotic cytokine (CNTF) promoted SOCS3 expression. Thus, in mice with non-induced (constitutive) increased SOCS3 expression, neither the STAT1 nor STAT3 pathways are able to reach the activation threshold to promote or inhibit apoptosis because of the inhibitory effect of SOCS3 on both pathways, which explains why specific pancreatic β-cell SOCS3 overexpression is unable to protect the mice against type 1 diabetes (35), despite its protective effects against cytokine-induced apoptosis. Conversely, pancreatic islet β-cells, which do not express SOCS3 (as in SOCS3 knock-out mice), show unregulated and hyperactive STAT1 and STAT3 pathways, which act independently from each other without SOCS3-mediated cross-talk. In this case, none of the pathways prevail, maintaining the cells in their previous non-apoptotic condition, thus preventing STZ-induced β-cell death.

However, the mechanism mentioned above is not valid for cytokine-induced physiological control of SOCS3 expression in β-cells. We demonstrated that inflammatory cytokines (IL1β)


Pancreatic Islets
iNOSKo mice
MIN6 Cells
+ 1mmol/l L-NAME
A
SOCS3
GAPDH
SOCS3/GAPDH
% of Control
0.1mg/kg CNTF
80mg/Kg STZ
F
1nmol/l CNTF
1mmol/l STZ
B
pSTAT1
STAT1
pSTAT1/STAT1
G
C
pSTAT3
STAT3
pSTAT3/STAT3
H
D
pIκB-α
IκB-α
IκBα/GAPDH
I
E
aCaspase3
Caspase3
aCaspase3/Caspase3
J

compete with anti-inflammatory (CNTF) cytokines to induce SOCS3 activation, although through distinct pathways, leading to opposing effects on β-cell apoptosis. IL1β promotes STAT1 phosphorylation, which increases SOCS3 expression and inhibits STAT3 phosphorylation, therefore inducing a proapoptotic and simultaneously inhibiting an anti-apoptotic pathway. When SOCS3 expression is reduced, STAT1-STAT3 pathways are dissociated, compromising both apoptotic and anti-apoptotic signals. Thus, it is clear that not only STAT1 and/or STAT3 activation promote their effects, but rather, it is the balance of the STAT1/STAT3 ratio that most promotes their effects. In other words, it is the STAT1/STAT3 ratio that determines β-cell life or death, as already proposed (36, 37). Super-expression of SOCS3 by ethanol inhibited the effects of cytokines on both the STAT1 and STAT3 pathways and their respective actions on human monocytes (38). In some cases, the effect of a single ligand, such as IFNγ, depends on the STAT1/STAT3 ratio, which determines the actions of cytokines on murine cells (39–41). Nevertheless, the present work is, to our knowledge, the first to propose and provide evidence that the STAT1/STAT3 ratio is a determining factor in pancreatic β-cell fate.

The protective effects of CNTF require not only STAT3 activation but also STAT3-induced STAT1 inhibition, through increased SOCS3 expression. In SOCS3kd mice and MIN6 cells, CNTF-induced STAT3 phosphorylation was actually increased when compared with WT mice and MIN6 cells; nevertheless, there was no inhibition of IL1β-induced apoptosis. The importance of the STAT1 pathway for β-cell apoptosis has already been observed (42), which is confirmed by the fact that STAT1 activation contributes to STZ-induced pancreatic β-cell apoptosis and the onset of type 1 diabetes in mice (43).

We also assessed the cellular mechanism by which STAT1 and STAT3 define β-cell life or death. STAT1 activates, whereas STAT3 inhibits, the NFκB pathway in pancreatic β-cells, which is essential for cytokine-induced apoptosis and type 1 diabetes (4). Whether IL1β alone activates the STAT1-NFκB pathway in pancreatic β-cells is still controversial. Here, however, we found a significant increase in STAT1 phosphorylation in pancreatic islets from STZ-treated mice, as well as IL1β-treated MIN6 cells, both accompanied by the activation of the NFκB pathway, which is in accordance with our previous findings (26).

In addition, CNTF required the inhibition of iNOS expression to promote its anti-apoptotic effects in mice pancreatic islets and MIN6 cells, as well as to inhibit STZ-induced type 1 diabetes. The importance of iNOS for STZ-induced type 1 diabetes in mice has been previously observed in a multiple low-dose streptozotocin model (44, 45). We confirmed these results in a single-dose (80 mg/kg) STZ-induced model of type 1 diabetes, and we also found that CNTF was unable to protect mice beyond the level found following the reduction of iNOS expression alone (iNOS KO mice), demonstrating that, despite the activation of upstream pathways, the effects of CNTF on β-cells invariably require iNOS down-regulation. All observations are thus summarized in Fig. 8. The similarity between the results obtained for pancreatic islets from STZ-treated mice and IL1β-treated MIN6 cells observed in the STAT-NFκB-iNOS pathway provide further evidence that IL1β is indeed the primary cytokine responsible for pancreatic β-cell death in type 1 diabetes, without ruling out the synergistic effects of other cytokines, particularly TNFα and IFNγ.

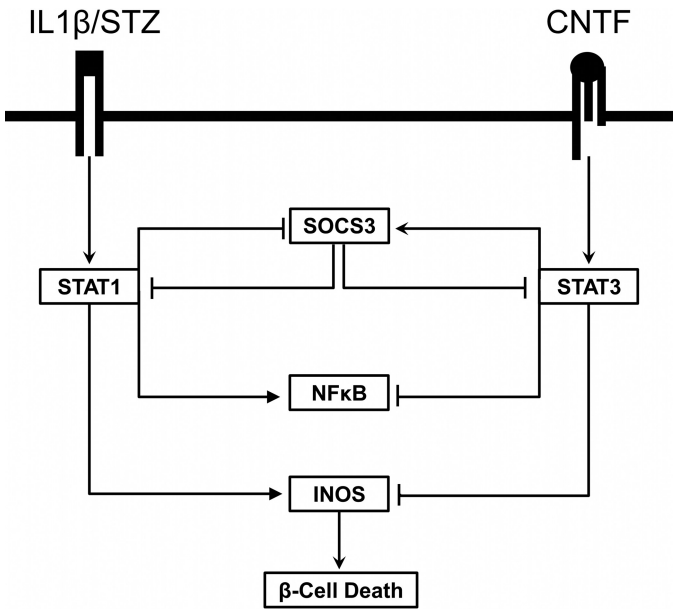


FIGURE 8. **Proposed mechanism for CNTF protection of MIN6 β-cells *in vitro* against IL1β-induced apoptosis and from STZ-induced pancreatic islets apoptosis *in vivo*.**

Finally, our results suggest that CNTF may be a potential pharmacological tool for the prevention and treatment of type 1 diabetes as well as for other inflammatory diseases, considering its anti-inflammatory characteristics. It could be argued that our experimental model required CNTF administration before the onset of the disease, therefore limiting its usefulness. It is important to remember, though, that the onset of type 1 diabetes in humans is a longer process, that, although usually detected in its final stages, could be delayed by CNTF administration, once diagnosed. CNTF could also be used in pancreatic islets from a donor to increase their viability and achieve a more efficient transplantation (46). This hypothesis is supported by the observation that SOCS3 delays pancreatic islet allograft rejection (34) and that, in human patients, inhibition of the NFκB pathway improves islet survival post-transplantation

FIGURE 7. **CNTF effects over pathways upstream to iNOS are unchanged, but it can no longer prevent STZ-induced apoptosis on islets from iNOSko mice or MIN6 cells treated with *N*-Nitro-L-Arginine Methyl Ester.** Shown are SOCS3 protein expression (*A*), STAT1 phosphorylation (*B*), STAT3 phosphorylation (*C*), IκB-α phosphorylation (*D*), and caspase-3 cleavage (*E*) of pancreatic islets from iNOSko mice. *White bars*, control; *gray bars*, CNTF; *hatched bars*, streptozotocin; and *black bars*, CNTF + streptozotocin ($n = 10$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF. †, significantly different from streptozotocin. Shown are SOCS3 protein expression (*F*), STAT1 phosphorylation (*G*), STAT3 phosphorylation (*H*), IκB-α phosphorylation (*I*), and caspase-3 cleavage (*J*) of MIN6 cells treated with citrate buffer (*white bars*), 1 nmol/liter CNTF (*gray bars*), 1 mmol/liter STZ (*hatched bars*), or both (*black bars*) ($n = 4$). Data are means ± S.E. *, significantly different from control; #, significantly different from CNTF; †, significantly different from STZ; $, significantly different from respective wild-type group.

(47). Another possibility is the use of CNTF to prevent β-cell mass loss in insulin-resistant patients before the onset of type 2 diabetes (48, 49) because SOCS3 controls β-cell mass (28) and CNTF increases β-cell mass in a type 2 diabetes mice model (50). Another advantage is that the approach used here could circumvent the generation of specific anti-CNTF antibodies observed in previous clinical trials (51), given that the dose is significantly lower and the period of treatment is shorter.

In conclusion, CNTF protects mice against STZ-induced type 1 diabetes, mainly by promoting survival of pancreatic islet β-cells, and this effect occurs through increased STAT3 phosphorylation, followed by increased SOCS3 expression and reduced STAT1 phosphorylation, which inhibits the NFκB pathway and reduces iNOS expression, inhibiting apoptosis. Moreover, we found that the STAT1/STAT3 ratio is most likely more important to determine β-cell death than the status of each STAT alone and that the STAT1/STAT3 ratio is controlled by SOCS3. Taken together, we provide strong evidence for another use of CNTF as an anti-diabetogenic tool.

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